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Relevance of RIBA-3 Supplementary Test to HCV PCR Positivity and Genotypes for HCV Confirmation of Blood Donors

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HCV antibody screening of 624,910 blood donations resulted in 3,832 samples being referred for confirmation. All were tested by RIBA-3 with 2,710 negative, 945 indeterminate and 177 positive results. HCV RNA was detected by PCR in an average of 69.5% of RIBA-3 positives (4 bands 84.1%; 3 bands 74.1%; 2 bands 34.1%) and only 0.53% of RIBA-3 indeterminates. Eighty-four percent of samples with a total RIBA-3 band intensity score (maximum 16) of ≥8 were PCR positive compared with only 22% of those with a score of <8. Total mean band intensities for HCV genotype 1 samples (n = 65) were 13.2, genotype 2 (n = 17) 11.4 and genotype 3 (n = 65) 11.2 with type 1 samples showing greater reactivity with c100 and c33 antibodies. No PCR positive type 1 samples were found with RIBA-3 total band scores less than 8, no PCR positive type 2 samples less than 6, whilst PCR positive type 3 samples were found with scores as low as 2. NS5 indeterminates were the most common (40.2%) single band pattern but yielded no PCR positive samples, followed by c33 (23.3%) with one PCR positive and c100 (20.2%) with one PCR positive whilst c22 indeterminates were least common (16.3%) but included three PCR positive donors. All five RIBA-3 indeterminate PCR positive donors were type 3.

KEY WORDS: ELISA, immunoblots, anti-HCV

INTRODUCTION

Screening of blood donors for hepatitis C virus (HCV) antibody commenced in the UK in September 1991 using second generation enzyme-linked immunoassays (ELISAs) incorporating recombinant antigens for three different regions of the HCV viral polyprotein (core-c22; NS3/NS4-c200). The Scottish National Blood Transfusion Service (SNBTS) policy for the confirmation of HCV infection was to test repeatedly ELISA reactive donations by the second generation recombinant immunoblot assay (RIBA-2) as a supplemental test [Dow et al., 1993]. Samples exhibiting any bands on RIBA-2 were further tested by polymerase chain reaction (PCR) for presence of HCV RNA. In late 1992, a third generation RIBA-3 superseded RIBA-2 as the supplemental assay. RIBA-3 incorporated several improvements including NS4 (c100) peptides in place of the original c100 and 5-1-1 recombinant proteins, c22 peptide instead of c22 recombinant protein, increased concentration of NS3 (c33c) recombinant protein and a new recombinant protein band from the NS5 region of the genome. This study examines the likelihood that RIBA-3 positivity can predict HCV viraemia (as measured by PCR) and also investigates the influence of HCV genotype on RIBA-3 results.

MATERIALS AND METHODS

ELISAs

During the study period, October 1992 to September 1994, the regional transfusion centres (RTCs) in Scotland and Northern Ireland used HCV ELISAs from three manufacturers: Abbott second and third generation assays (Abbott GmbH, Delkenheim, Germany), Ortho second (2.5) and third generation (3.0) assays (Ortho Diagnostics, Raritan, NJ) and Murex VK48 (Versions I, II and III) (Murex Diagnostics, Dartford, UK). Murex Version II was introduced in December 1993 and incorporated a modification to the NS4 antigen whilst Version III was introduced in April 1994 and included a truncated NS5 antigen. All assays were carried out according to the manufacturer's instructions. Repeatedly ELISA reactive samples referred from the RTCs were all tested by RIBA-3 and HCV ELISAs from the two other manufacturers.

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TABLE I. Comparison of RIBA-3 Positive Patterns With Presence of HCV Viraemia and HCV Genotype in 177 RIBA-3 Positive Blood Donors

	RIBA-3 positive patterns						HCV Type					
	c100	c33	c22	NS5	Number	PCR Positive	1	2	3	4	5	Untypeable
4 Bands	+	+	+	+	82	69 (84.1%)	41	7[a]	27	1	1	5
3 Bands	+	+	+	−	39	25	15	2	11	1	0	10
	+	+	−	+	2	2	1	0	1	0	0	0
	+	−	+	+	4	4	0	1	3	0	0	0
	−	+	+	+	9	9	0	4	5	0	0	0
					54	40 (74.1%)	16	7	20	1	0	10
2 Bands	+	+	−	−	7	3	4	0	0	0	0	3
	+	−	+	−	10	2	0	1	3	0	0	6
	+	−	−	+	4	0	0	0	0	0	0	4
	−	+	+	−	16	9	3	2	8	0	0	3
	−	+	−	+	1	0	0	0	0	0	0	1
	−	−	+	+	3	0	1	0	2	0	0	0
					41	14 (34.1%)	8	3	13	0	0	17
Total	148	156	163	105	177	123 (69.5%)	65	17	60	2	1	32

[a]One sample reactive for both HCV types 2 and 3 by serology.

Immunoblots

The recombinant immunoblot assay RIBA-3 (Chiron Corporation, Emeryville, CA) uses two recombinant proteins (c33c, NS5) and two synthetic peptide antigens (c100p and c22p) covering four regions of the viral polyprotein (core, NS3, NS4 and NS5). Tests were carried out according to the manufacturer's instructions. Reactivity to an individual antigen was quantified by comparing with internal control band intensities incorporated in each strip, i.e., a 1+ control band was scored as 1+, a 3+ control band as 3+, a band greater than 1+ and less than 3+ as 2+, and a band greater than 3+ control as 4+ intensity.

A further immunoblot assay INNOLIA-3 (Innogenetics NV, Zwijndrecht, Belgium) was used to test selected donations. This test used both synthetic peptides (core, NS1, NS4, NS5) and a recombinant protein (NS3) independent of the Chiron assay system.

PCR

The polymerase chain reaction (PCR) was carried out on donor plasma using nested primers for the 5′ region of the HCV genome as previously described [Chan et al., 1992]. More recently a commercial amplicor (Roche Diagnostics, Branchburg, NJ) HCV RNA test was also used in parallel with the in house PCR system.

HCV Genotyping

HCV genotyping was undertaken on all PCR positive samples by restriction fragment length polymorphism (RFLP) examination of restriction endonuclease-treated PCR products as described by Davidson et al. [1995]. Serological typing for HCV genotypes 1–6 was performed on all RIBA positive PCR negative samples using a prototype HCV serotyping ELISA based on genotype specific NS4 peptides [Bhattacherjee et al., 1995], similar to a commercially available assay (Murex Diagnostics).

RESULTS

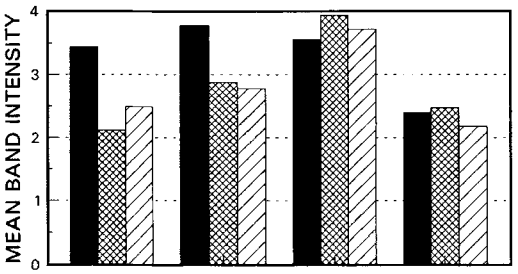
During the 2 year period, October 1992 to September 1994, 3,832 samples were recieved from RTCs for anti-HCV confirmation. RIBA-3 testing of these samples showed 177 (4.6%) referrals to be positive (≥2 bands), 945 (24.6%) to be indeterminate (i.e., 1 band) and the remaining 2,710 to be negative. Of the RIBA-3 positives, 123 (69.5%) were PCR positive whilst only five (0.53%) of the RIBA-3 indeterminates tested PCR positive.

RIBA-3 Positives

The commonest band found amongst RIBA-3 positive samples was c22 (92.1%) followed in order by c33 (88.1%), c100 (83.6%) and NS5 (59.3%). Amongst the 123 PCR positive samples, 118 (96%) were c22 positive, 117 (95%) c33 positive with either of these bands being found in all 123 RIBA-3 and PCR positive samples. The presence of anti-c100 (85%) or anti-NS5 (68%) was lower amongst the RIBA-3 PCR positive group.

The number of reactive RIBA-3 bands and patterns of reactivity were correlated with HCV viraemia and PCR genotype (Table I). HCV viraemia was greatest amongst samples reactive with all 4 RIBA-3 bands (84.1%) and lowest in those reactive with only 2 RIBA-3 bands (34.1%). Examination of RIBA-3 positive samples exhibiting 3 bands showed all samples containing NS5 band were PCR positive. In contrast, among the six possible combinations of 2 band patterns in RIBA-3, only three were associated with PCR positive samples—none of these contained NS5.

Sera from donors infected with different genotypes showed significantly different patterns of reactivity by the RIBA-3 assay. Type 1 samples frequently showed reactivity to all four antigens (63%), while type 3 less often (45%). Whilst type 1 was the most common genotype (53%) amongst RIBA positive samples with all 4



RIBA Band	c100	c33c	c22	NS5
TYPE 1 (N=65)	3.44	3.78	3.56	2.40
TYPE 2 (N=17)	2.12	2.88	3.94	2.47
TYPE 3 (N=65)	2.49	2.78	3.72	2.18

Fig. 1. Mean RIBA-3 band intensity according to HCV type in all typeable samples excluding two HCV type 4s and one type 5.

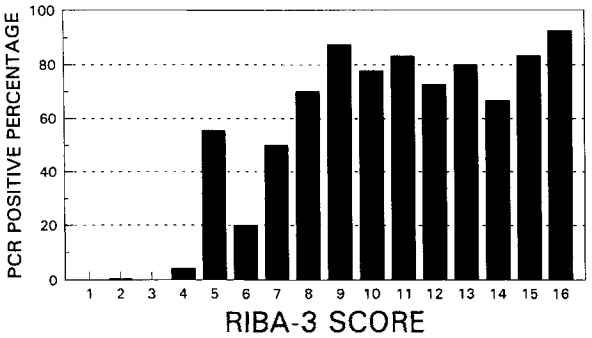


Fig. 2. Comparison of HCV PCR activity with total RIBA-3 band intensities score for all RIBA-3 reactive sera (including indeterminates).

bands, type 3 was the commonest genotype amongst RIBA positives with 3 (45%) and 2 (54%) bands.

Type 1 samples also showed significantly greater intensities for c100 and c33 (Fig. 1). If mean band intensities for the 4 RIBA-3 antigens are added together (max = 16) the type 1 samples (n = 65) showed the strongest reactivity with a mean score of 13.2, while type 2 (n = 17) and type 3 (n = 65) samples reacted more weakly (mean scores of 11.4 and 11.2 respectively).

The relationship between PCR status and the total score of RIBA-3 band intensities is shown in Figure 2. Although the percentage PCR positivity increased with RIBA score there was not a linear relationship, nor was there any RIBA score where 100% PCR positivity was apparent. For total scores of less than 8, only nine (21.9%) of 41 RIBA-3 positives were PCR positive whereas for scores of 8 or greater, 114 (83.8%) of samples were PCR positive. No type 1 samples were PCR positive with a RIBA-3 score of less than 8; no type 2 samples were PCR positive with a RIBA score of less than 6, whilst PCR positive type 3 samples were found with as low a score as 2 and several were RIBA-3 indeterminates (see below).

A marked reduction in the frequency of PCR positives was observed between those reacting with 3 antigens in RIBA-3 compared with those reacting with only 2. All samples exhibiting reactivity with 2 RIBA-3 bands were further investigated by testing in third generation HCV ELISAs and by a further immunoblot assay Innolia-3. All 15 PCR positives within this group were clearly reactive in all HCV ELISAs (Ortho-3, Abbott-3, Murex VK 48 version III) and Innolia-3, while the 27 PCR negative samples in this group gave variable results. A total of 17 reacted in all four tests, one sample with c100 and c33 bands and 16 others all reactive with c22 band plus another band (6 c100, 7 c33 and 3 NS5). The other 10 samples gave varying ELISA results (Table II). None were reactive by the prototype HCV serotyping ELISA. Six samples were completely negative with all three ELISAs. Three of the remaining four reacted by only a single ELISA (2 Abbott, 1 Murex) and a single sample by two ELISAs (Abbott-3, Ortho-3).

Apart from one sample, all RIBA positives reactive with 3 or 4 bands were reactive in all manufacturers ELISAs. The single exception failed to react by either Abbott and Ortho second generation ELISAs, yet was PCR positive and clearly detected by third generation tests [Dow et al., 1994]. This sample was type 3 and reacted in all versions of the Murex ELISA. It showed reactivity to 3 RIBA-3 bands—c100 (1+), c33 (3+) and NS5 (4+). Testing by alternative immunoblot, Innolia, showed only a 3+ intensity NS5 banding pattern. A sample taken 3 months later had identical results.

RIBA-3 Indeterminates

Despite NS5 indeterminates being the most common (40.2%) indeterminate banding pattern none were shown to be PCR positive; c33 indeterminates were next most common (23.3%) with one PCR positive; c100 with 20.2% and one PCR positive; whilst c22 indeterminates were the least common (16.3%) banding pattern yet included three PCR positives.

In total five (0.5%) RIBA-3 indeterminate samples were PCR positive (Table III) and all were shown to be type 3 by RFLP. All but the c33 PCR positive sample were detected clearly with all second and third generation ELISAs. This one sample exhibited only a 2+ intensity with the RIBA-3 c33 band, was reactive in the Ortho-3 ELISA, but negative in Abbott-3, Murex VK48 screening assays and also Innolia-3 supplementary test. Further samples 3 months and 1 year later from this donor gave identical results.

DISCUSSION

The aim of this study was to correlate RIBA-3 results with the presence of HCV viraemia and HCV genotype. The data indicate that viraemia is related to the number of reactive bands in the RIBA-3 test and the total score for band intensity (each band scoring from 0 to 4). However, the correlation was not sufficiently high to allow for the reliable prediction of PCR positivity directly from the RIBA-3 pattern or total score. The analysis of the data was further complicated by the observation that type 3 and to a lesser extent type 2 may not be reacting optimally in RIBA-3. Modification of RIBA-3 will be nec-

TABLE II. Alternative HCV Tests Performed on 10 Selected Blood Donations Reactive to Only 2 Bands in RIBA-3 But Shown to Be HCV PCR Negative

Donor specimen	RIBA-3 c100	RIBA-3 c33	RIBA-3 c22	RIBA-3 NS5	INNOLIA III	HCV ELISA Abbott 3.0	HCV ELISA Ortho 3.0	HCV ELISA Murex VK48 version III	Initially reactive in
T11388 E	3+	3+	–	–	Neg	Neg	Neg	Neg	Murex vI
T12344 E	1+	2+	–	–	Neg	Neg	Neg	Neg	Murex vI
T8570 E	2+	–	1+	–	Ind[a]	Neg	Neg	Neg	Murex vI
T11852 E	2+	–	1+	–	Ind[a]	Neg	Neg	Neg	Murex vI
T9408 E	2+	–	–	2+	Neg	Neg	Neg	Neg	Murex vI
T11755 G	2+	–	–	2+	Neg	+	+	Neg	Abbott-2
T11892 E	1+	–	–	1+	Neg	Neg	Neg	Neg	Murex vI
T18278 B	1+	–	–	2+	Neg	+	Neg	Neg	Abbott-3
T20661 E	–	2+	2+	–	Neg	Neg	Neg	+	Murex vIII
T8908 G	–	3+	–	1+	Neg	+	Neg	Neg	Abbott-2

[a]NS4 1+ band only.

TABLE III. Comparison of RIBA-3 Indeterminate Banding Intensities and HCV PCR Activity in Blood Donations Exhibiting Single Bands in RIBA-3

RIBA-3 Band	Band intensity	Number of donations	HCV PCR positive
c100	1+	68	0
	2+	51	0
	3+	50	0
	4+	22	1
		191	1
c33c	1+	61	0
	2+	59	1
	3+	51	0
	4+	49	0
		220	1
c22	1+	52	0
	2+	49	0
	3+	35	0
	4+	18	3
		154	3
NS5	1+	160	0
	2+	128	0
	3+	75	0
	4+	17	0
		380	0
Totals		945	5 (0.53%)

essary to provide optimal detection and in addition, our data also suggest that the need for an ELISA including type 3 specific antigens should be investigated.

Adding the score of the RIBA-3 band intensities demonstrated that a total score of 8 or greater was associated with an 84% chance of viraemia compared to only a 22% chance in samples with a score of 7 or less. Furthermore, PCR positive results were infrequent in samples infected with type 1 with a RIBA-3 score of less than 8 and in type 2 infected blood donors with a score of less than 6. That PCR positive results were found at lower RIBA-3 scores only with type 3 samples and that all PCR positive RIBA-3 indeterminates were type 3 indicate that RIBA-3 is less sensitive for this genotype, particularly for antibody against the NS3 and NS4 antigens. We found previously that RIBA-2 is even less sensitive for type 3 with weak or absent antibody for NS3 and NS4 antigens [McOmish et al., 1993, 1994]. The presently constituted RIBA-3 test has higher reactivities to types 2 and 3 but still lower than type 1. Additional type 2 and 3 specific components may therefore be necessary to improve the reactivity of RIBA-3 to non-HCV type 1 genotypes. This may be particularly relevant in countries of South Asia where type 3 is the major HCV type [Davidson et al., 1995] and increasing numbers of sub-types are being recognised.

The low frequency of viraemia found amongst 2 band RIBA-3 positive samples could have been due to resolving HCV infection but could also be attributed to false positive results. It is apparent that some 2 band RIBA-3 positive PCR negative samples may react in only one of the currently available third generation ELISAs and that some detected by earlier generations of tests may react in none of the current tests. Consequently, 2 band RIBA-3 positive samples should be investigated with all available tests including PCR before making a diagnosis of HCV infection. Damen et al. [1995] have also suggested that a number of such samples might be non-specific as interviewing a limited number of 2 band RIBA-3 positive donors revealed only 18% with risk factors for HCV infection whereas 83% of 3 and 4 band RIBA-3 positive donors had risk factors.

That only five of 945 RIBA-3 indeterminate samples were found PCR positive in the 2 year period of this study and that all were type 3 is reassuring for the reliability of blood donor screening and confirmation in countries where types 1 and 2 are the exclusive or dominant types as it indicates that the RIBA-3 test is likely to be positive with all viraemic samples. The c33 indeterminate PCR positive sample demonstrated again the higher reactivity of the Ortho HCV 3.0 ELISA and RIBA-3 test for this particular antibody which often develops first in infection [Courouce et al., 1994a, 1994b; Goffin et al., 1994; Vrielink et al., 1995]. Whilst RIBA-3 c100 indeterminate PCR positive blood donors are rare, the one found in this study was detected by all HCV

ELISAs and also the alternative immunoblot. Zaaijer et al. [1994] have suggested that c100 antigen should be removed from HCV antibody assays to enhance their specificity. This particular donor was shown to have similar results 1 year later and removing c100 antigen from current assays would lead to donors of this type being missed.

From the accumulated data in a country where genotypes 1, 2 and 3 are circulating we would conclude that a combination of current ELISAs (based on type 1 recombinant antigens) and RIBA-3 (based on type 1 recombinant antigens/peptides) can provide a very effective method of screening and supplementary testing of blood donors in countries where only types 1 and 2 are present. Problems are apparent with some type 3 samples and these need to be addressed by the manufacturers. The data therefore suggest that PCR should be retained as a confirmatory test where there are significant numbers of blood donors infected with HCV type 3.

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